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COMBINATIONS AND VARIATIONS OF TECH-
NIQUES FOR IMPROVED CHROMOSOME STUDIES
IN THE GRAMINEAE

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(WITH PLATE I)

Cytologists today emphasise the superiority of the squash method over the microtome technique for the study of chromosomes in most species of plants. In general, however, a certain amount of difficulty is experienced in obtaining good somatic metaphase plates from the root tips of grasses and cereals. The root tips are hard and difficult to squash due to the failure of the cells to separate readily from one another, even after prolonged hydrolysis in N.HCl, or repeated heating in the acetic stains.

By combining various methods and techniques it has been possible to obtain rather good results for chromosomal investigations in all the species of pasture grasses and cereals tested by the author.

PRETREATMENT BEFORE FIXATION.

Many plant species especially the *Gramineae* have fairly large chromosome numbers. In order to make accurate counts and morphological studies it has been found necessary to contract the chromosomes and scatter them over a larger cell area than the normal metaphase plate. Amongst the first chemicals tried was chloral hydrate (Kagawa, 1929). With the discovery in 1937 by Blakeslee and Avery, and Nebel and Ruttle,

that colchicine induces polyploidy in plants, it was shown that this organic substance inhibits spindle formation, contracts the chromosomes and in a large number of plant species causes a scattering of the chromosomes within the cell. O'Mara (1939) recommended the use of colchicine for the studies of grass chromosome morphology. In many grasses, however, colchicine caused a clumping of the chromosomes. Later investigations led to the discovery of a number of treatments which produced effects similar to that of colchicine. Swanson (1940) used acenaphthene. Östergren (1944) described a number of c-metaphase inducing substances. Meyer (1945) gave a paradichlorobenzene treatment, and Hill and Myers (1945) a cold treatment for 24 hours to facilitate chromosome counting. O'Mara (1948) made an extensive study of a large number of organic compounds and recommended the use of monobromonaphthalene and monobromobenzene for metaphase and 3 per cent methanol treatment for prophase chromosome studies in *Zea*. Tjio and Levan (1950) preferred oxyquinoline, and Caroli (1952) oxyquinoline derivatives.

During the present study it was found that the majority of cereals and grasses responded very well to either colchicine or monobromonaphthalene pretreatment or both. In most cereals the results were equally good—a 0.05 per cent colchicine solution acting quicker than a saturated aqueous solution of monobromonaphthalene. For maximum chromosome contraction, in order to facilitate their counting, the optimum periods of pretreatment at 20° to 25°C were 3 hours with the former and 3½ to 4 hours with the latter. These two compounds were preferred to oxyquinoline—although Tjio and Levan (1950) claim that it reveals the structure of the centomere and secondary constrictions much better—because it caused a fuzzy chromosome contour. According to Bhaduri and Ghosh (1954), who likewise preferred the use of monobromonaphthalene, the introduction of oxyquinoline to the fixative had an effect similar to the oxyquinoline pretreatment in exaggerating the centromeres and secondary constrictions. The presence of oxyquinoline in the fixative has the disadvantage that it produces a suspension of dark staining fatty particles in the tissues (Bhaduri and Ghosh, 1954).

FIXATION.

The acetic acid fixatives (Carnoy and modifications) are widely used because they are inexpensive, easy to use, penetrate quickly, fix rapidly, do not stain the cytoplasm and to a certain extent dissolve the middle lamella. Root tips are often more readily squashable, especially after the Feulgen staining schedule. However, in the *Gramineae* the chromosomes were very often badly fixed in these fixatives, having a "bubbly"

appearance and fuzzy outlines obscuring the clarity of the centromeres and secondary constrictions. They frequently also caused a certain amount of chromosome stickiness. Furthermore they did not harden and coagulate the cytoplasm sufficiently, so that especially after hydrolysis in N.HCl, the tapping and squashing of the root tips to separate the tenaciously bound cells, caused them to break so that bits of cytoplasm with some chromosomes floated away. Similar results were obtained when using the methyl alcohol-acetic acid fixative of O'Mara (1948), ethyl alcohol-propionic acid combinations (cf. Johansen, 1940; Sparrow and Sparrow, 1949), and methyl alcohol-propionic acid mixtures with and without chloroform. (However, in some Liliaceous plants, e.g. *Ornithogalum*, mixtures consisting of 6 parts methyl alcohol : 3 chloroform : 2 propionic acid, or 3 methyl alcohol : 2 propionic acid gave results far superior to 3 : 1 or 6 : 3 : 1 Carnoy).

The osmic and Navashin fixatives, without further chemical treatment, were rather useless for squash preparations in most *Gramineae*. Not only were the root tips harder and the cells more tenaciously bound together, but they also caused staining and severe granulatory coagulation of the cytoplasm, which greatly reduced the chromosome contrast. This is a great pity, since these, especially the osmic fixatives, are generally the best for chromosome fixation (cf. La Cour, 1947). Flovik (1938) has convincingly shown that La Cour's 2BD fixative produced the best chromosome fixation when studied in microtomed sections. Bhaduri and Ghosh (1954) similarly recommended osmic Benda.

In order to prevent excessive hardening and blackening, La Cour (1953) advised that after 1 hour fixation in 2BD, an equal volume of 1 per cent chromic acid in distilled water should be added to the fixative. The root tips are to remain in this mixture overnight, and are then thoroughly washed in distilled water.

La Cour (1947) bleached the stained roots in a mixture consisting of 1 part 20 vol. hydrogen peroxide and 3 parts 80 per cent alcohol for 3 to 6 hours. Bhaduri and Ghosh (1954) washed the root tips in dilute sulphuric acid, and during dehydration treated the preparation with a 1 : 1 mixture of redistilled turpentine and n-butyl alcohol.

MACERATION.

A number of different treatments have been suggested to improve cell separation without influencing the staining capacity of the chromosomes. The majority of these were directed at the breakdown and dissolution of the pectinaceous middle lamella; others were used to digest the cellulose cell wall.

Hillary (1939) recommended a 15 min. treatment at 60°C in 4 per

cent (by volume) NH_4OH , or 40 min. in 4 per cent ammonium oxalate at 60°C to break the pectic salts of the middle lamella down to soluble pectate, which can be removed by washing. Fabergé (1945) gave an over-night treatment with snail stomach fluid, containing an active mixture of cytase, to dissolve the cell walls of the root tips. McKay and Clarke (1946) used a 1 per cent solution of "Pectinol", obtained from *Aspergillus*, with success. Hohl (1948), in order to macerate soft plant parts, treated the tissues with a 1 to 5 per cent solution of the proprietary enzyme, "Pectinol W", made by Rohm and Haas, buffered to pH 5.0 with acetic acid. After deaeration in an aspirator the tissues remained in the solution for 15 to 24 hours at 28° to 30°C . Pectic enzymes derived from various *Aspergillus* and *Penicillium* species were also used with great success. After a 24 hour extraction period and filtration, a 5 per cent solution of another commercial enzyme complex, "Filtragol", made by Bayer Leverkusen, containing a large percentage of pectinase, was used for 3 hours by Bauch and Overbeck (1949). Although a pH 6.0 was regarded optimal for the enzyme, the solution was not buffered since the root tips were considered sufficiently acid after fixation. Chayen (1949) similarly used a pectic enzyme derived from *Penicillium digitatum*, but since the maceration potencies of different samples varied, Chayen and Miles (1954) preferred the use of the standardised commercial enzyme, "Pectinase", made by Nutritional Biochemical Corp., Cleveland, Ohio. To inhibit the proteolytic enzymes, a 5 per cent solution (by weight) of the powder was extracted in a 1 per cent aqueous solution of peptone. Maceration for 1 hour in this solution was sufficient for material fixed in acetic-alcohol. Harris and Blackman (1954) treated the root tips for 12 hours in a 2 per cent "Pectinase" solution adjusted to pH 6.6. Ford (1952) gave a treatment of ammonium oxalate-hydrogen peroxide after osmic fixation to bleach the cytoplasm and dissolve the middle lamella.

With all the *Gramineae* investigated by the present author, a solution of pectinase was found to be most suitable for macerating root tips. A commercial cytase preparation tried, adversely affected the Feulgen staining. The pectinase solution was prepared by extracting 0.1 gm. of the commercial "Pectinase" (Nutritional Biochemical Corp., Cleveland, Ohio) in 10 c.c. distilled water or 1 per cent peptone, for at least 30 min. at 30°C . and filtering. Excellent pectinase solutions can also be prepared by extracting pectic bran on which *Aspergillus niger* was cultivated—0.5 gm. bran per 10 c.c. distilled water. It was not found necessary to adjust the pH, but this would probably be advantageous. In order to control microbial contamination Harris and Blackman (1954) added toluene to the enzyme preparation, but Hohl (1948) found that 0.05 to 0.1 per cent merthiolate was more effective.

The enzyme treatment for complete separation of the cells at 30°C. was about 18 hours after Carnoy fixation, 24 hours after 2BD fixation, and even longer after Navashin fixation. By using stronger enzyme concentrations, the optimum pH, and deaeration, the treatment can be shortened. Excellent maceration were achieved in this way after all fixatives. By gently tapping the root tip with the flattened back of a bone needle the cells fall apart while the contents of each cell remains intact. However, as previously mentioned the cytoplasm was too dark and granular after osmic and Navashin fixation to allow any critical chromosome observations when Feulgen squash preparations were made. After Carnoy fixation the staining was intense and the cytoplasm clear; the drawback here was the poor fixation of the chromosomes, and the softness of the cytoplasm after digestion. With some species, however, very good results could be obtained.

The ammonium oxalate-hydrogen peroxide technique (Ford, 1952) was found very useful in many plant species after 2BD and Benda osmic fixatives for clearing the cytoplasm and macerating the root tips. In most of the *Gramineae* a treatment of up to 15 min. at about 20°C was not sufficient to produce satisfactory separation of the cells. Prolonged treatment softened the tissue too much and severely reduced the stainability of the chromosomes.

A combination of the ammonium oxalate-hydrogen peroxide and pectinase treatments in the end proved to be the most satisfactory. After 2BD, Benda and Navashin fixation, and the Feulgen staining method, excellent separation of the cells, clear cytoplasm and well fixed, intensely stained chromosomes were obtained. Furthermore the pectinase treatment period was very much reduced.

After fixation the root tips were thoroughly washed in distilled water, and treated for 10 min. in a 1 : 1 mixture of saturated ammonium oxalate in distilled water and 20 vol. hydrogen peroxide. Although the 1 per cent pectinase treatment can also be given after staining, it is generally given prior to hydrolysis. According to the species involved the middle lamella pectin was adequately broken down after 8 to 12 hours at room temperature, or 5 to 7 hours at 30°C.

The advantage of this method is that single whole cells at the metaphase stage can readily be obtained (Plate 1a-d), the chromosomes of which can be further spread apart, if necessary, by applying slight pressure on the coverslip overhead between two thicknesses of blotting paper, and very gently heating the slide over a spirit flame.

To summarise, the following schedule has been used for the critical investigation of the chromosomes from seedling root tips of various species of grasses and cereals:

- (1) Germinate the seeds between sterile, well soaked, thick filter paper. When the roots have reached a length of 0.25 to 0.5 cm. place the seedlings between damp filter paper for 1 or 2 days at 25°C.
- (2) Cut off the roots about 1 cm. from the tip and place these in small glass vials containing a saturated aqueous solution of monobromonaphthalene, or a 0.05 per cent colchicine solution. The best time to start the pretreatment is between 9.00 and 11.00 a.m.
- (3) After 3½ to 4 hours in the monobromonaphthalene, or 3 hours in the colchicine solution at 25°C., fix the roots in La Cour's 2BD fixative for 1 hour.
- (4) Add an equal quantity of 1 per cent chromic acid in distilled water to the fixative and leave overnight.
- (5) Rinse the roots thoroughly in distilled water and leave in distilled water for 30 min. (Change the distilled water every 5 to 10 minutes).
- (5a) Store in 70 per cent alcohol if necessary.
- (6) Decant the distilled water and place the roots in a mixture consisting of 1 part saturated aqueous solution of ammonium oxalate and 1 part 20 vol. (=6%) hydrogen peroxide for 10 min. in strong light in order to bleach and for preliminary maceration.
- (7) Wash the roots thoroughly in distilled water (5 changes over a 30 min. period will suffice).
- (8) Place the roots in the macerating enzyme solution (0.1 to 0.5 gm. "Pectinase" is added to 10 ml. distilled water or 1 per cent peptone, extracted for at least 30 min. at 30°C. and filtered. Buffer the solution to pH 6.0 with acetic acid, and add toluene or 0.005 to 0.01 gm. merthiolate crystals). Deaerate if necessary. Allow the enzyme to digest for 5 to 7 hours at 30°C. or 8 to 12 hours at room temperature.
- (9) Rinse the roots in water.
- (10) Hydrolyse the roots in N.HCl at 60°C. for 12 to 15 min.
- (11) Stain in Leuco-basic-fuchsin (as prepared by La Cour, 1947) for 2 to 3 hours.
- (11a) If necessary intensify the colouration by washing the roots in tap water for 5 to 30 min.
- (12) Cut off the meristematic root tip in a drop of 45 per cent acetic acid or aceto-carmin and gently tap the tissue with the flattened handle of a bone or plastic needle until the cells are well separated.
- (13) Cover with an albuminised cover slip and gently give a few more taps. Examine under the microscope, and after locating the cells

at the required stage, apply slight pressure on the cover slip between two thicknesses of blotting paper to flatten the cells and scatter the chromosomes. Warm the slide very gently if necessary—too much heat can spoil the whole preparation.

- (14) To make the preparation permanent, separate the slide and cover slip by inverting the slide in a ridged dish containing 45 per cent acetic acid.
- (15) Pass the cover slip through the following series, remaining 1 to 2 minutes in each: 60 per cent alcohol plus a few drops of acetic acid; 80 per cent alcohol, 95 per cent alcohol; absolute alcohol; 1 absolute alcohol: 1 Euparal essence (can be omitted).
- (16) Recombine the slide and cover slip by mounting in Euparal.

NOTE ON P.M.C. FIXATION.

During his investigation of *Ornithogalum* (*Liliaceae*) the author had difficulty with "bubbly" fixation of the chromosomes, and stickiness of both chromosomes and P.M.C.'s when the ordinary acetic fixatives were used. A modification of O'Mara's (1948) methyl alcohol fixative was tried and was found to be far superior. This fixative was later also used on grass and cereal P.M.C.'s with gratifying results (Plate 1 e-h). The bivalents were well fixed, the propionic or aceto-carmin (or a mixture of the two) staining was intense, the tendency of the P.M.C.'s to stick together was greatly reduced and the elasticity of the cytoplasm was such that it could be pressed out of the cell wall without breaking apart or losing its circular shape.

The fixative consists of 6 parts methyl alcohol: 3 parts chloroform: 2 parts propionic acid with a trace of ferric chloride added. Fix for 24 hours and store either in the fixative or preferably in 70 per cent alcohol in a refrigerator. Best squashing results are obtained after a 4-day storage period. With some material the chloroform can be omitted. Preparations are made permanent as described in the above schedule.

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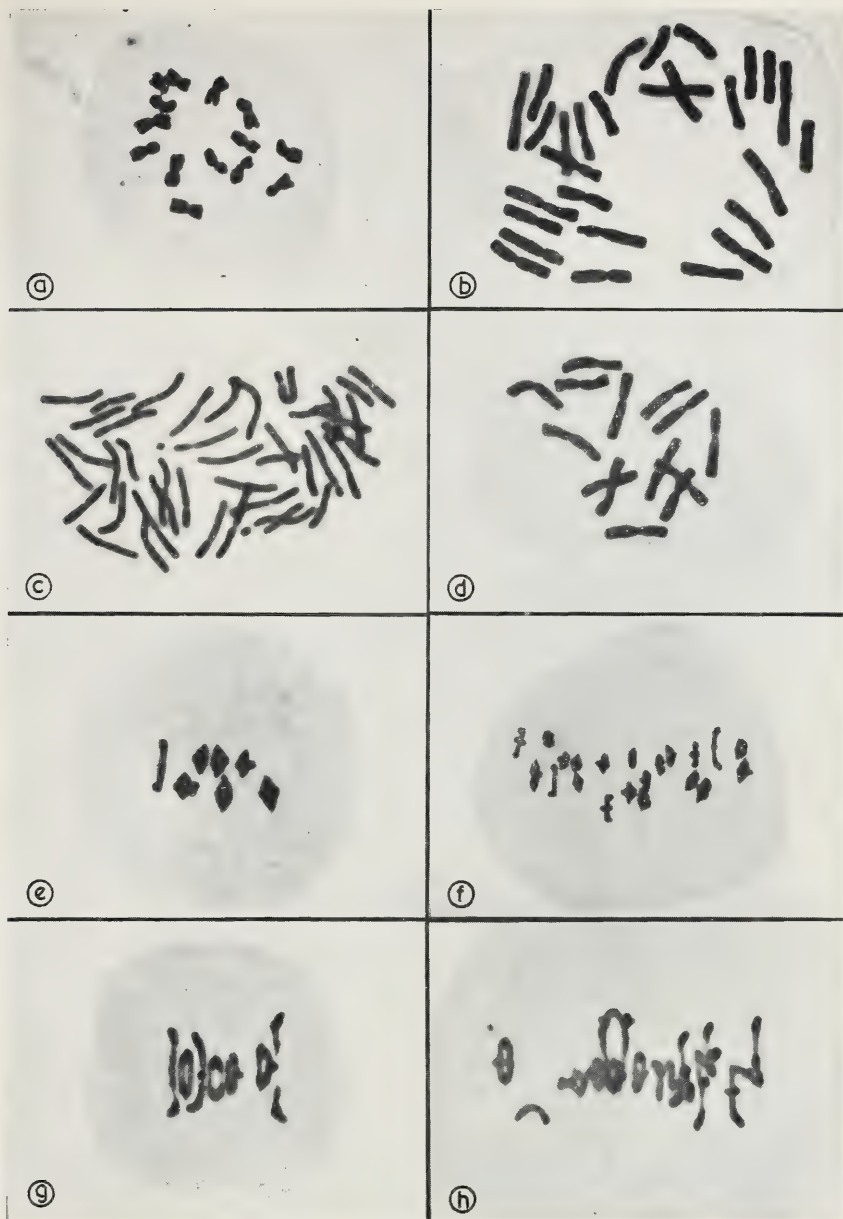


PLATE 1: Photomicrographs of Feulgen (a—d) and aceto- and propionic-carmin (e—h) preparations. The photomicrographs were taken with a Carl Zeiss, Jena, microscope and Zeiss Winkel attachment camera to which a Leica camera was fitted, on Kodak Microfilm film which was developed in Kodak D.158 developer for 3 minutes at 20°C. and fixed in "Amfix", made by May and Baker Ltd., for 2 min. Figs. a and b were taken from temporary preparations whereas figs. c to h were taken from permanent preparations.

- (a) *Pennisetum glaucum* root tip chromosomes, x 1645.
- (b) *Agropyron distichum* root tip chromosomes, x 1645.
- (c) *Triticum vulgare* root tip chromosomes, x 1645.
- (d) *Secale cereale* root tip chromosomes, x 1645.
- (e) *Festuca elatior* P.M.C. at MI, x 1000.
- (f) *Festuca arundinacea* P.M.C. at MI, x 725.
- (g) *Secale cereale* (diploid) P.M.C. at MI, x 847.
- (h) *Secale cereale* (pentasomic tetraploid) P.M.C. at MI, x 847.